Screen-printed digital microfluidics combined with surface acoustic wave nebulization for hydrogen-deuterium exchange measurements

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FULL TITLE: Screen-Printed Digital Microfluidics Combined with Surface Acoustic Wave Nebulization for Hydrogen-Deuterium Exchange Measurements

SHORT TITLE: A novel chip-based hydrogen-deuterium exchange workflow (68 characters)

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Keywords: Digital Microfluidics / Surface acoustic wave nebulization / Hydrogen/Deuterium exchange

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RATIONALE (2-3 sentences, approx. 50 words):

METHODS (max 75 words):

RESULTS (up to75 words):

CONCLUSIONS (1 or 2 sentences, approx. 50 words):

An inexpensive digital microfluidic (DMF) chip was fabricated by screen-printing electrodes on a sheet of polyimide. This device was manually integrated with surface acoustic wave nebulization (SAWN) MS to conduct hydrogen/deuterium exchange (HDX) of peptides. The HDX experiment was performed by DMF mixing of one aqueous droplet of angiotensin II with a second containing various concentrations of D_2O. Subsequently, the degree of HDX was measured immediately by SAWN-MS. As expected for a small peptide, the isotopically resolved mass spectrum for angiotensin revealed that maximum deuterium exchange was achieved using 50% D_2O. Additionally, using SAWN-MS alone, the global HDX kinetics of ubiquitin were found to be similar to published NMR data and back exchange rates for the uncooled apparatus using high inlet capillary temperatures was less than 6%.

Hydrogen deuterium exchange (HDX) is a powerful technique for studying protein structure [1]. As evidenced by the rapidly growing body of HDX literature, HDX has seen steady growth. For the most part, HDX workflows have deviated little from HPLC or a direct infusion apparatus coupled to ESI-MS. In this study, we investigate the use of two fundamental changes to the HDX workflow. First, we examine the use of an alternative ionization method called surface acoustic wave nebulization (SAWN) and second, we explore the use of microfluidics as an alternative to manual or an automated LC-type unit for sample preparation.

While ESI has been critical to HDX success and many other MS based assays [2,3], it is not without limitations. For instance, while ESI is highly sensitive, it can lead to in-source fragmentation and/or the oxidation of small molecules and proteins [4–6], and ESI requires its own charged, continuous flow apparatus. To address the analytical short-comings of ESI, many alternative ionization techniques have been developed, such as Desorption Ionization on Silicon [7], Desorption-ESI [8] and Laser Ablation-ESI [9].

In this study, we employed surface acoustic wave nebulization (SAWN) for HDX analysis. SAWN generates ions from a planar piezoelectric surface and delivers them to the inlet of a mass spectrometer [10]. To accomplish this, an alternating current is applied to interdigitated transducers (IDT, interlocking electrodes) on a piezoelectric $LiNbO_3$ wafer to generate a high frequency surface acoustic wave [11]. When the SAW reaches an area on the surface of the chip where an aqueous droplet of sample is located reflection of the wave within the droplet results in nebulization of the liquid sample within seconds. To date, a range of analytes of small molecules have been analyzed by SAWN-MS [5,6,10].

SAWN has several advantages over ESI and other ionization techniques for HDX. First, SAWN has been found to generate ions of lower energy than ESI [6], which has the potential to maintain the structural integrity of more analytes during the ionization process [5]. Additionally, given its relative “softness” compared to ESI, there is the potential advantage of reduced back exchange during HDX, which we test here. Second, in contrast to ESI, SAWN is very simple to operate with no possibility of clogging since it is a planar substrate. Specifically, SAWN operation only involves transferring a droplet of sample directly onto the chip and activation of the chip, which leads to the immediate nebulization of the sample. In this paper, we validated the use of HDX via SAWN-MS using ubiquitin, a well-characterized protein [12–14].

In addition to the ionization source, we explored the use of a sophisticated fluid handling platform called digital microfluidics (DMF) for sample manipulation within the HDX workflow. The concept of DMF is representative of a class of techniques using the relatively weak interactions between electric fields and polarizable droplets of liquid on a planar surface. A typical DMF device consists of a flat substrate covered in a microelectrode pattern which is covered by subsequent layers of a suitable dielectric and a hydrophobic layer [15]. This technology has been referred to by many names during its development including; metal-insulator-solution-transport [16], digital microfluidic system [17], electrowetting on insulator coated electrodes [18], electrowetting on dielectric [19] and finally DMF [20].

Due to the promise of automated sample handling, small sample volumes, loss-less sample preparation, and miniaturized devices that can forgo large and costly LC systems, much effort has been placed into connecting microfluidics with ESI-MS as seen in the publication data presented in these review articles [21,22]. However, coupling ESI-MS to microfluidic systems remains a challenge due to the requirement of maintaining constant fluid flow for steady electrospray [23]. The most popular means for coupling microfluidics with electrospray is simply attaching a micro-electrospray emitter [21], such as the microfluidic bottom-up HDX device manufactured by Rob et al. [24]. This microfluidic device has successfully characterized HDX on very short timescales with low back exchange [25,26]. However, this device has several potential disadvantages such as time-consuming fabrication (due to attachment of a sprayer and incorporation of microchannels) and susceptibility to clogs (especially when using native buffer). Microfluidics has also been coupled with MALDI-MS to circumvent these problems, but MALDI is susceptible to matrix effects [27]. Alternatively, integrating SAWN and DMF is simple and conserves sample since both are planar platforms which manipulate discrete droplets as low as half a microliter [20].

Here, we demonstrate the ease of use of DMF-SAWN to perform HDX on an inexpensive DMF device. Briefly, the DMF device was made by screen-printing conductive ink on top of a flexible polyimide substrate and coating the device with hydrophobic materials. The motivation for utilizing an inexpensive DMF design was to obviate the problem of biofouling by making the device disposable. We report results obtained from fusing droplets of D_2O and angiotensin II on such a disposable DMF device and analyzing the sample immediately by SAWN-MS.

The significance of this report is three-fold. Firstly, it represents the first use of SAWN to ionize HDX samples for MS analysis. Secondly, we demonstrated that SAWN-MS can yield high-resolution, reproducible data for whole proteins with low back exchange. Thirdly, SAWN was successfully coupled with a disposable, screen-printed DMF device to provide a means for sample preparation at the mass spectrometer. This combination points the way toward monitoring more complex chemical reactions in real time by DMF-SAWN-MS with many different types of analytes for a new, sample conserving "lab on a chip" or micro total analysis system [22,28].

2 Materials and Methods

2.1 Fabrication and Operation of SAWN Chips. Fabrication of SAWN chips has been reported in detail previously [6,10]. In summary, a SAW transducer consisting of 20 pairs of 100 μm interdigitated (IDT) electrodes (40 in total) with 100 μm spacing and 10 mm aperture, along with a secondary electrode to apply external electrical potential, were patterned onto the surface of 128 Y-cut X-propagating 3 inch $LiNbO_3$ wafers purchased from Crystal Technology, Inc. (Palo Alto, Ca). The SAWN configuration was first designed in AutoCAD before being written into a chrome mask by a Heidelberg µPG 101 Laser Pattern Generator (Heidelberg Instruments Mikrotechnik GmbH Tullastrasse 2, D-69126 Heidelberg, Germany) at the University of Washington Nanotech User Facility (https://depts.washington.edu/ntuf/). Wafers were then coated using AZ 1512 positive photoresist (AZ Electronic Materials, Somerville, NJ) spun at 4000 rpm for 30 seconds creating a 1-1.2 μm thick resist layer. Exposure of the photo resist was done for 5 seconds using an Oriel mask aligner (Newport Corporation, CA). The exposed wafers were placed in a development bath for 60 seconds in AZ 351 (AZ Electronic Materials, Somerville, NJ). To pattern conductive IDT electrodes a 20 nm chrome adhesion layer was deposited by heated vapor deposition followed by a 60 nm layer of gold, followed by lift-off in acetone for 30 minutes. The resulting SAWN IDT has a resonance frequency of 9.56 MHz. To operate the SAWN chip a MXG analog signal generator (Agilent N5181A, Santa Clara, CA) and a Mini Circuits ZHL-5W-1, 5-500 MHz RF amplifier (GwInstek GPS-2303, New York, NY) were used to generate and amplify the RF signal.

2.2 Fabrication of Screen-Printed DMF Chips. Flexible, disposable, DMF chips were produced in a proprietary low-cost printing process using carbon-containing conductive ink on

50 μm thick flexible polyimide foils (DuPont Kapton) to pattern electrodes with 100 μm spacing, connection leads and external contact pads, followed by a 7 μm layer of ink acting as dielectric layer. After the printing process, a fluoropolymer solution, either Teflon-AF (DuPont) or Cytop (Asahi Glass) solution was spin-coated onto the devices and left to dry in ambient condition to create a 150 nm thick hydrophobic layer. The finished devices could be stored in normal laboratory conditions over the time period of several months without influence on performance.

2.3 Operation of Screen-Printed DMF Chips. A schematic showing the operation of the DMF is shown in **Figure 5**. Droplets were moved, merged and transferred to the SAWN chip in AC mode [28,29] with a driving voltage of 500 Vpp and a frequency of 20 kHz. The devices performed flawlessly and without any noticeable degradation over several experimental cycles. The electrode geometry used was an arrangement of two parallel columns of square electrodes. The droplet was held between two adjacent electrodes from the respective columns by applying the driving voltage between them. To move the droplet to the next electrode pair, the driving voltage was first applied to the new pair, pulling the droplet in between the pairs, and then switching off the voltage between the first pair. DMF operation was controlled using control software running on a PC that was connected to custom drive electronics. For this, the 2.5 Vpp AC sine output of a 20 kHz signal generator was amplified by a high-voltage amplifier (Trek) to 500 Vpp and distributed to the DMF electrode pads using a distribution circuit based on solid-state relays. Connection to the DMF devices was through 2.54 mm pitch edge connectors.

2.4 HDX of Ubiquitin. In an eppendorf tube, 1µl of 200 µM ubiquitin, 40 mM ammonium bicarbonate (pH 7.4) with 19 µl of D_2O for a final concentration of 10 µM ubiquitin, 2mM ammonium bicarbonate. Exposure to deuterium was varied so as to obtain many time points to properly visualize the rate of deuterium uptake in ubiquitin over time. To quench the reaction, 1.5 µl of 10% formic acid was added to the mixture. 3 spectra were obtained of three replicates of the same time point. To obtain a mass spectrum of the mixture, 1 µl of the quenched reaction mixture was placed on the SAWN chip surface in front of the interdigitated transducer. An ultrazoom scan of this droplet was recorded with a thermo LTQ-Velos using the s-lens at 60% voltage and a capillary temperature of 200C after activating the SAWN chip with 25 dBm at 9.56 Mhz. To calculate the overall percent deuteration, the percent deuteration of the +6, +7, and +8 ion was calculated as per the equation 1 reported in Zhang and Smith [30] with the centroided mass obtained using HX express [31,32], and then these values were averaged for the final percent deuteration value. Estimated percent deuteration was achieved by using the method described in Pan et al. [13] and the rate constants from Bougalt et al. [12] adjusted for pH differences.

2.5 Measuring Back Exchange of Ubiquitin. As before, 1µl of stock solution was pipetted into 19µl of D_2O. The reaction mixtures was boiled for 10 min. and then dried by speed vac. Then, 19µl of D_2O and 1.5µl of 10% formic acid was added to the dried sample, mixed briefly, and then pipetted onto the SAWN chip for analysis by SAWN-MS. Back exchange was estimated according to the following formula:

$$\frac{MW_{measured} - MW_{undeuterated} - X_{fast} - X_{inexchangeable}}{X_{backbone}}$$

$MW_{measured}$ is the centroid measured molecular weight (in Da), $MW_{undeuterated}$ is the average mass of ubiquitin (8564.8449Da), X_{fast} refers to the number of side-chain exchanges in ubiquitin (72), $X_{inexchangeable}$ refers to the number of amides that exchange only extremely slowly in native conditions (21) [14], and $X_{backbone}$ is the total number of backbone exchanges possible in ubiquitin (73).

2.6 HDX Mass Spectrometry of Angiotensin. 4 µL droplet of angiotensin II (10µM, Proteomass) in H_2O and a 4 µL droplet of D_2O were pipetted onto separate regions of the flexible DMF chip. Then, an AC field to the electrodes adjacent to the droplets was applied to transport and fuse the two droplets. The fused droplets were transferred directly from the hydrophobic DMF chip to the hydrophilic SAWN chip by DMF and wetting. After transfer, SAWN was activated to aerosolize the droplet and HDX was monitored by a Thermo LTQ linear ion trap.

3 RESULTS AND DISCUSSION

3.1 HDX of Ubiquitin. To demonstrate the utility of SAWN for HDX analysis of proteins, global HDX studies were carried out on ubiquitin, a well characterized protein. High-resolution mass spectra showing the isotopic envelope of deuterated ubiquitin were readily obtained by SAWN-MS on an ion trap mass spectrometer, and the mass spectra clearly demonstrate a shift of the ubiquitin ions towards higher m/z values with increased exposure to D_2O (**Figure 1**). Due to these promising results using SAWN for HDX, we set out to record a full time course of the HDX reaction by SAWN to show that SAWN-HDX can be used to rigorously measure global HDX rates. These results are displayed in **Figure 2**. Each data point represents triplicate measurements that highlight the reproducibility of this technique. Additionally, the experimental per-deuterated time-point of 94.6% is very close to the maximum possible 95% deuteration of ubiquitin under these conditions. Most importantly, however, the data from SAWN-MS correlates well with global estimated percent deuteration values based on HDX-NMR data obtained by Bougault, especially for the later time points [12] (**Figure 2**). An additional point of validation is that the results of Pan et al. [13] compare well to the SAWN-MS data. Pan et al. measured a global percent deuteration of 83% at 30 min. of incubation for ubiquitin under similar conditions. When a simple natural log regression is fitted to the SAWN-MS data (r-squared value 0.9957), the estimated percent deuteration measured by SAWN-MS with 30 min. incubation would be 80.4%, which agrees well with Pan et al. [13]. Finally, the back exchange of the SAWN-HDX setup was measured. At room temperature HDX carried out by hand, very low rates of back exchange were measured that were either lower or close to the value reported by Rob et. al. (**Figure 3**) [26], depending on the inlet capillary temperature.

3.1 DMF Chip Fabrication. Though SAWN chips are simple to operate they remain rudimentary in their ability to accommodate fluid handling or chromatography. To address this issue we developed a technique for fabrication of low-cost screen-printed DMF chips on polyimide that can easily be coupled with SAWN and may be considered disposable (**Figure 4**). These inexpensive DMF chips were prepared by screen-printing [33,34] on to flexible polyimide substrates. Specifically, screen-printed electrodes were developed using a carbon-containing conductive ink on top of which was printed a dielectric with a layer of hydrophobic Cytop on top of these first two layers.

3.2 HDX via DMF-SAWN-MS. HDX was carried out on the signal peptide angiotensin II to demonstrate the functionality of DMF-SAWN-HDX. The movement of droplets on the DMF chip and transfer to SAWN is shown in **Figure 5.** The design of the DMF chip used a series of electrodes spaced 100 µm apart (**Figure 5A**). Samples were pipetted onto the surface of the DMF chip across from an equal volume of D_2O as shown in **Figure 5B**. Once both droplets were in place on the DMF chip, the sample droplet consisting of a 4 µl droplet of angiotensin II in H_2O was actuated towards the 4 µl droplet of D_2O by applying a field on the adjacent electrode while simultaneously releasing the field on the initial electrode. In this way, one can actuate droplets back and forth across the DMF chip and combine droplets to initiate HDX as shown in

Figure 5C. Transfer of the post-HDX reaction sample to the SAWN chip was performed by placing the droplet in contact with the surface of the SAWN wafer, which allowed the droplet to wick easily over to the natively hydrophilic lithium niobate SAWN chip from the more hydrophobic coated Cytop DMF chip **(Figure 5D-E**).

The mass spectra of angiotensin II before (**Figure 6A**) and after a one minute incubation (**Figure 6B**) to allow HDX to occur are shown in **Figure 6**. In addition to the expected $[M+H]^+$ ion of angiotensin II, the presence of a small amount of sodiated angiotensin II, $[M+Na]^+$, is observed. The post-HDX mass spectrum is shown in **Figure 6B** which shows that eight deuteriums were found to be present on angiotensin II, which is the maximum possible deuteration for angiotensin II in 50% D_2O. These results are expected for a small peptide like angiotensin II (1.046 KDa), since all the sites are highly solvent accessible.

4 Concluding Remarks

In summary, we have demonstrated the use of SAWN-MS for monitoring HDX reactions of small proteins. Additionally, and importantly for future automation of the method, we have shown it is feasible to combine a DMF sample preparation chip with the SAWN chip to carry out HDX monitoring by MS. The DMF chips were produced by screen-printing onto a flexible, polyimide substrate making the chip effectively disposable. These HDX methods using SAWN-MS and DMF-SAWN-MS offer a new, facile means of elucidating protein structure. Given the simplicity of these methods, we envision them being used to carry out pilot studies rapidly at the MS interface.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

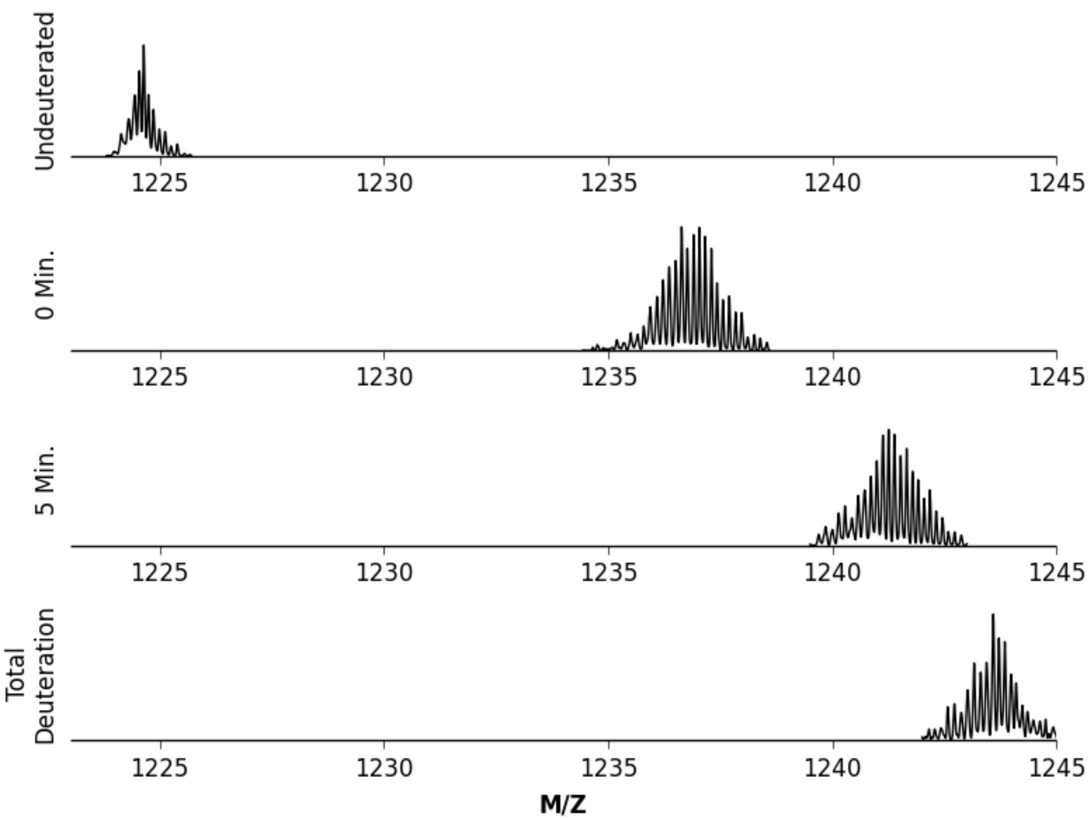

Figure 1. SAWN-MS Monitored HDX of Ubiquitin. The zero minute mass spectrum is obtained from ubiquitin exposed to quench solution. The five minute mass spectrum was obtained from ubiquitin exposed to D_2O for five minutes. The totally deuterated spectrum was obtained by boiling in D_2O for 10 minutes. All spectra were collected by an LTQ-Velos in ultrazoom scan mode.

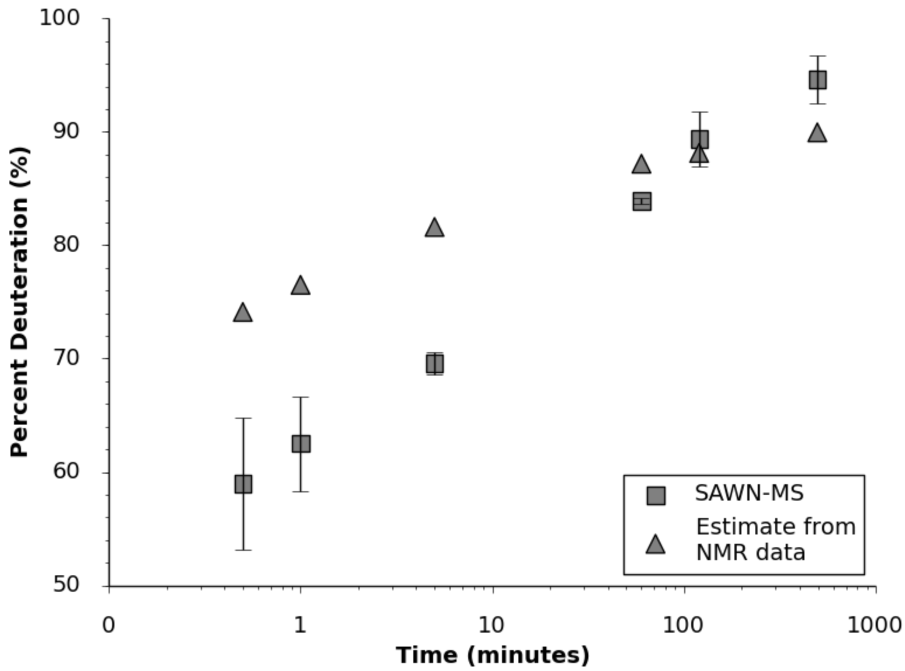

Figure 2. Time-course of Deuterium Uptake in Ubiquitin. Time refers to the incubation time of ubiquitin in 95% D_2O at room temperature. The blue squares represent the experimentally derived percent deuteration. This was obtained by calculating the percent deuteration for each of the +6, +7, and +8 with the aid of equation #1 [30] and HX-express [31,32] and averaging these values. Error bars represent the standard deviation for the average of the three replicates of the percent deuteration levels found with each of the three charge states. The red triangles represent the estimated global percent deuteration based on combining NMR kinetic data of ubiqutin [12] with the equation by Pan et al. [13].

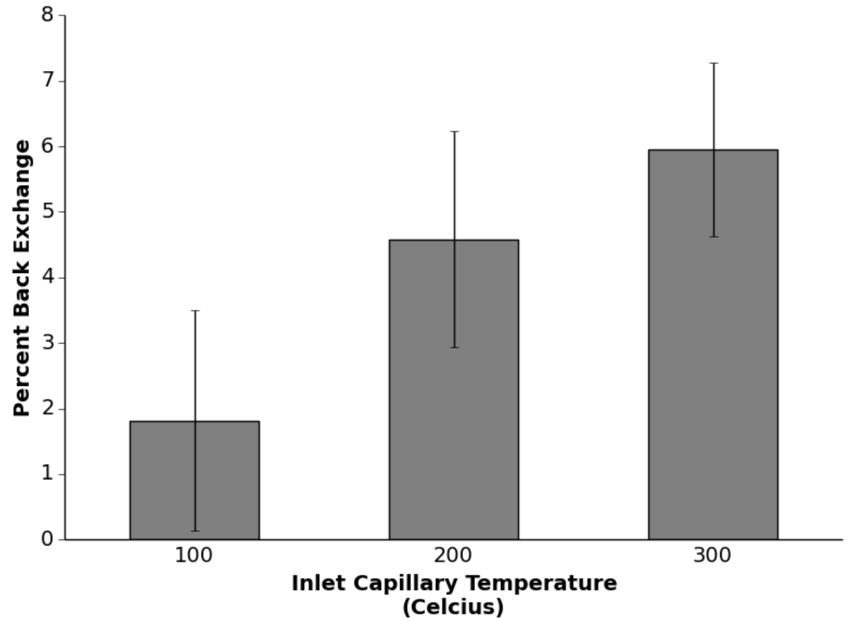

Figure 3. SAWN-MS Measured Back Exchanged. Percent back exchange obtained from SAWN-MS HDX at room temperature with various inlet capillary temperatures of ubiquitin. Back exchange values for each temperature are based on the average of three different samples.

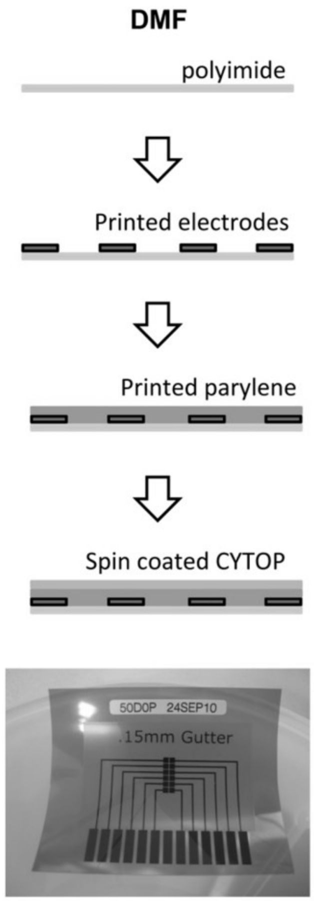

Figure 4. Fabrication of Digital Microfluidic (DMF) chips. DMF chips were fabricated by screening printing conductive ink onto 50 μm thick flexible polyimide foils.

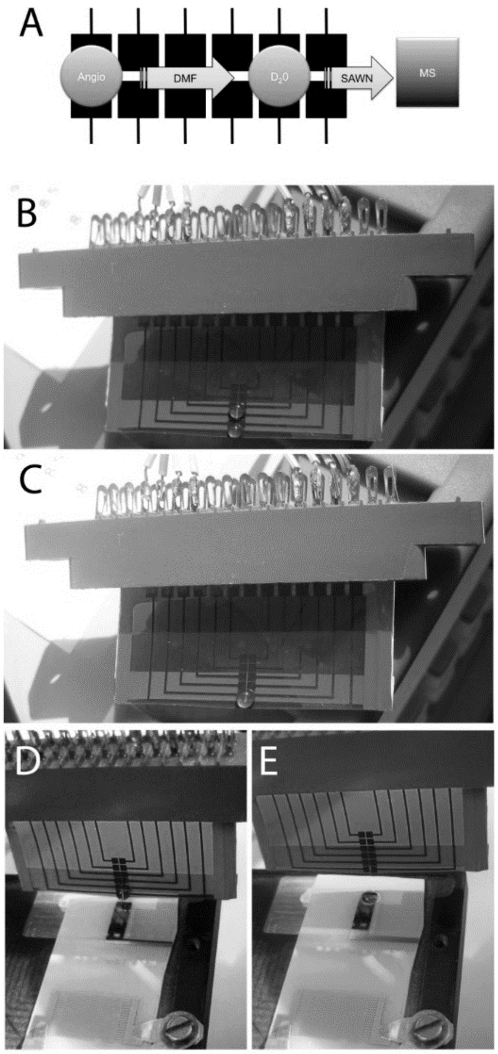

Figure 5. DMF Experimental Workflow. The basic HDX workflow is shown in **(A)**. Droplets were pipetted onto the surface of the DMF chip **(B)** where they were moved by applying an AC field to adjacent electrodes until adjacent droplets combine **(C).** After being combined the droplet was transferred from the hydrophobic DMF chip to the natively hydrophilic lithium niobate SAWN wafer **(D-E)** by wicking action.

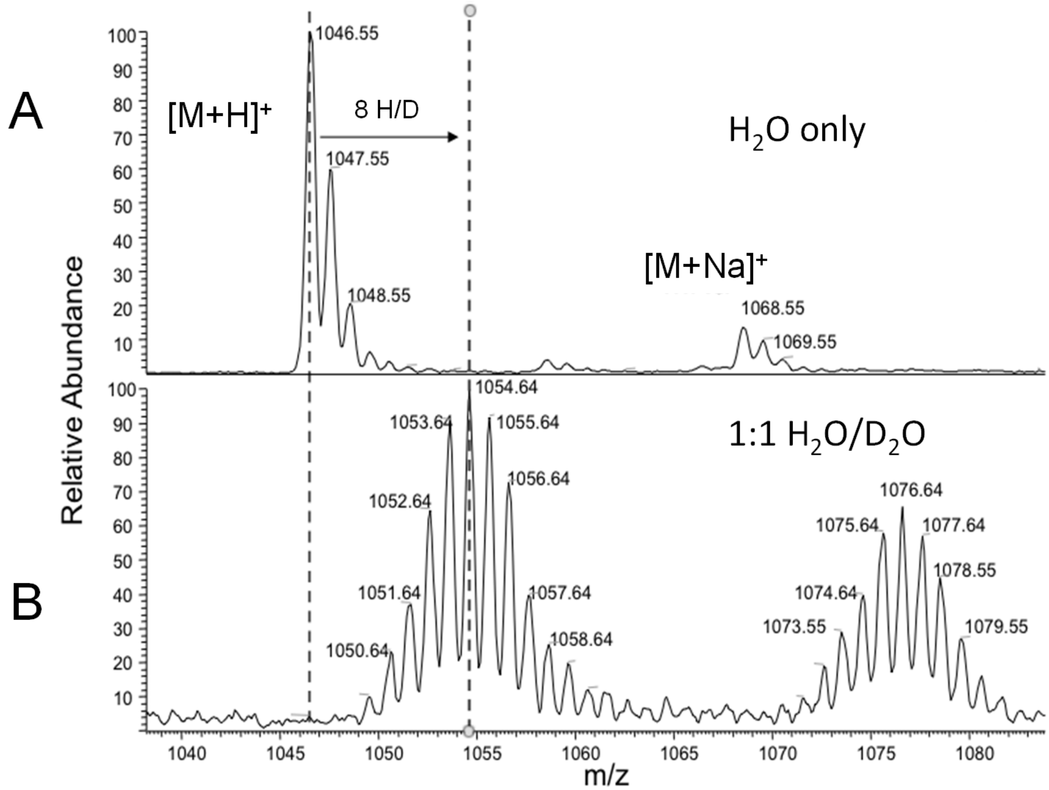

Figure 6. HDX of angiotensin II Measured by DMF-SAWN-MS. An angiotensin II spectrum is shown prior to HDX **(A)** and one minute post- mixing **(B)** by DMF manipulation of a droplet of angiotensin in water with a droplet of D_2O. One minute of exposure of angiotensin II in 50% D_2O resulted in 8 deuterium exchanges.